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The experimental determination of reliable biodegradation rates for mono-aromatics towards evaluating QSBR models

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ABSTRACT

Quantitative Structure Biodegradation Relationships (QSBRs) are a tool to predict the biodegradability of chemicals. The objective of this work was to generate reliable biodegradation data for mono-aromatic chemicals in order to evaluate and verify previously developed QSBRs models. A robust biodegradation test method was developed to estimate specific substrate utilization rates, which were used as a proxy for biodegradation rates of chemicals in pure culture. Five representative mono-aromatic chemicals were selected that spanned a wide range of biodegradability. Aerobic biodegradation experiments were performed for each chemical in batch reactors seeded with known degraders. Chemical removal, degrader growth and CO_2 production were monitored over time. Experimental data were interpreted using a full carbon mass balance model, and Monod kinetic parameters (Y, K_s, q_{max} and μ_{max}) for each chemical were determined. In addition, stoichiometric equations for aerobic mineralization of the test chemicals were developed. The theoretically estimated biomass and CO_2 yields were similar to those experimentally observed; 35% (s.d ± 8%) of the recovered substrate carbon was converted to biomass, and 65% (s.d ± 8%) was mineralised to CO_2. Significant correlations were observed between the experimentally determined specific substrate utilization rates, as represented by q_{max} and q_{max}/K_s, at high and low substrate concentrations, respectively, and the first order biodegradation rate constants predicted by a previous QSBR study. Similarly, the correlation between q_{max} and selected molecular descriptors characterizing the chemicals structure in a previous QSBR study was also significant. These results suggest that QSBR models can be reliable and robust in prioritising chemical half-lives for regulatory screening purposes.

1. Introduction

Reliable prediction of chemical biodegradation rates can help prioritise evaluation efforts on chemicals that pose the greatest risk to the environment and humans. QSBR models link predictor variables, predominantly physiochemical properties, to response variables that function as associated biodegradation indices of the chemical (e.g., half-life, ThOD, BOD_5, rate constants) (Raymond et al., 2001; Wammer and Peters, 2005; Pavan and Worth, 2008; Rücker and Kümmerer, 2012; Xu et al., 2015). Their use in persistence, bioaccumulation and toxicity (PBT), and other ecotoxicological assessments of chemicals can reduce the need for experimental tests on animals and their associated costs (Höfer et al., 2004; Martin et al., 2017a). However, the acceptance of QSBR model predictions depend on their reliability and relevance (Nendza et al., 2013). According to Annex XI of the Registration, Evaluation, Authorisation & Restriction of Chemicals (REACH) directive, the use of a QSAR (Quantitative Structure Activity Relationship) model for regulatory purposes is valid if: (i) the model is developed in accordance to OECD principles, (ii) the evaluated substance is within the applicability domain of the model, (iii) the predicted result is suitable to use for regulatory purposes, and (iv) adequate documentation of the method is provided (Nendza et al., 2013; Echa, 2016).

In our previous work (Acharya et al., 2019), QSBR models for 60 simple mono-aromatic chemicals were developed in accordance with OECD principles. The models related molecular descriptors to the natural logarithm of first order biodegradation rate constants. These first-order biodegradation rate constants were calculated

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using semi-quantitative biodegradation data encompassed in the BIOWIN3 model (US-EPA, 2012), which are based on the ratings given by experts to evaluate the relative biodegradability of chemicals and are unlikely to represent true biodegradation rates. The biodegradation data included in BIOWIN models are derived from ready biodegradability tests (RBTs). However, RBTs exhibit a number of limitations that are particularly pertinent to their use in persistence assessment (Kowalczyk et al., 2015). These limitations include (i) high levels of variation (inter-replicate, inter-test, inter-facility and temporal), (ii) a large number of test fails, (iii) an arbitrary time restriction (the pass criterion is reached in a 10 days window within the 28 days test period; the test pass criteria being -70% removal of Dissolved Organic Carbon, DOC, and 60% of Theoretical Oxygen Demand, ThOD, or Theoretical Carbon dioxide, $ThCO_2$, production), and (iv) the lack of a requirement to provide information on rates of biodegradation, or parameters related to such rates (Kowalczyk et al., 2015). RBTs are pass/fail tests that provide no accurate information on rates or half-life endpoints used for persistence assessments. The qualitative data produced by RBTs thus needed regression models to convert them into quantitative half-life data, the accuracy and reliability of which could be questioned (Arnot et al., 2005; Acharya et al., 2019).

Reliable estimation of biodegradation kinetics depends on the test protocol, the experimental system used for the biodegradation assay, and the kinetic models used to fit the experimental data. Before attempting to predict biodegradation rates (or their associated indices; e.g. half-lives, rate constants, substrate utilization rates) from the structure of chemicals for environments with complex biology (e.g., natural and engineered ecosystems), it would be informative to explore how well the biodegradability of chemicals can be predicted from their structures in more constrained systems, where biological complexity is minimised. Therefore, a good starting point is to work with well-defined constrained systems with known degraders at high inoculum load to reduce biological complexity. In such a system, the probable acclimation effect of a chemical in microbial growth is minimised, while variation in observed biodegradation results can be reduced to obtain reproducible rates. In contrast, biodegradation rates obtained from experiments performed with mixed consortia (e.g., activated sludge inocula) might be difficult to interpret due to complexity, microbial anonymity, and sample variation of inocula used. Experimental rates (e.g. substrate utilization rates) from simplified systems can be fitted to a number of models (such as the Monod kinetic model), which enable estimation of the biodegradation/biotransformation rate constants of specific chemicals (Simkins and Alexander, 1984; Pitter and Chudoba, 1990; McCarty, 2012).

The main objective of this study was to generate substrate utilization rates from batch experiments with pure cultures of degraders to validate the principles of a previously developed QSBR models (Acharya et al., 2019) for simple aromatic chemicals. To ensure that the measured substrate utilization rates from batch experiments with pure culture are reliable, and to explore potential uncertainties in their experimental determination, biomass concentrations and CO_2 were monitored in parallel with the substrate degradation, and a carbon mass balance model was developed to rigorously interlink these experimental observations when interpreting the data with respect to Monod kinetics. While the carbon mass balance does not reduce the uncertainty in the experimental data, it can reduce uncertainty in the fitting of multiple parameters from the data, because it establishes a quantitative relationship between the observed changes in the three variables; substrate, biomass and CO_2 concentration. To add further rigour, the experimentally determined biomass yields were compared to theoretical predictions based on stoichiometric equations for microbial growth on each single substrate, to provide in-depth understanding of the biodegradation process and ensure universality. Finally, the validation and verification of a previously developed QSBR model (Acharya et al., 2019) was performed by conducting a correlation analysis between the biodegradation rates predicted by QSBR model and the experimentally determined specific substrate utilization rates.

2. Material and methods

2.1. Chemicals and bacterial strains

Five test chemicals were selected from a previous chemical dataset (Acharya et al., 2019), by considering three criteria: 1) the representation of a wide range of biodegradation rate constants (i.e. 1st order rate), 2) the availability of detailed aerobic biodegradation pathways, and 3) use of a minimal number of microorganisms in biodegradation assays so that the confounding factors due to different physiologies would be reduced. Based on the aforementioned criteria, phenol, 2,4-dichlorophenol, 4-chlorophenol, m-cresol and toluene were selected. Chemicals with known aerobic biodegradation pathways having the highest (phenol), lowest (2,4-dichlorophenol) and intermediate (4-chlorophenol, m-cresol and toluene) biodegradation rates were selected from the dataset for this study. In general, the biodegradation of these chemicals can occur with one unique pathway (e.g., 2,4-dichlorophenol) and/or multiple pathways (e.g., 4-chlorophenol, phenol, toluene and m-cresol) depending upon the type of degrader and environmental conditions (i.e. aerobic and anaerobic) (Zylstra et al., 1988; Gao et al., 2010; Arora and Bae, 2014). The details of probable aerobic biodegradation pathways of these chemicals are described elsewhere (Gao et al., 2010). All chemicals were >99% pure (Sigma-Aldrich, Dorset, United Kingdom). Pure cultures with the metabolic capability to mineralize these chemicals were chosen as an inoculum for the batch biodegradation experiments and were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) (Table 1). Upon arrival, all the bacterial strains were reactivated and stored in the prescribed media (i.e. nutrient broth medium (1 g/L glucose, 15 g/L peptone, 6 g/L NaCl, 3 g/L yeast extract; pH 7.5 ± 0.2) according to the supplier's instructions. Growth studies of each bacterium were performed in nutrient broth medium.

2.2. Experimental system and biodegradation assays

Experiments were carried out in reactors consisting of a 500 mL Duran Schott glass bottle, modified on the side and the top as shown in Fig. 1 (Final volume; 580 mL, please refer **Section 8 in** Supporting **Information for more details**). The biodegradation studies for each of the chemicals were carried out, one at a time, in the above batch reactors (Fig. 1). The inoculum for each biodegradation experiment was taken during the exponential growth phase from a culture growing on nutrient broth medium. Approximately 10^7 cells/mL, quantified by flow cytometry (Section 4 **in SI**), were inoculated aseptically into each sterile reactor bottle pre-filled with 200 mL sterilized minimal microbial growth medium (6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl) (Sigma – Aldrich, United Kingdom) containing 0.2 mL of trace element solution (Section 1 **in SI**). Prior to inoculation, bacteria were centrifuged at $5000 \times g$ for 10 min at room temperature and washed twice with phosphate buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4; pH 7.3). To maintain a constant pH in the reactor, 10 mM of sterile HEPES buffer (Sigma-Aldrich, United Kingdom) was added to the minimal media. 50 mg/L of test chemical was spiked into the reactor, which was then capped and

Table 1
List of chemicals, their known degrader strains and their designation.

Chemicals	Degrader	Culture collection
Phenol	*Pseudomonas putida* F1 (Reardon et al., 2000)	DSMZ (6899)
Toluene	*Pseudomonas putida* F1 (Spain and Gibson, 1988; Zylstra et al., 1988; Reardon et al., 2000)	DSMZ (6899)
m-Cresol	*Pseudomonas putida* F1 (Spain and Gibson, 1988)	DSMZ (6899)
4-Chlorophenol	*Cupriavidus baselensis*. (Steinle et al., 1998; Vandamme and Coenye, 2004)	DSMZ (11853)
2,4-Dichlorophenol	*Cupriavidus baselensis*. (Steinle et al., 1998; Vandamme and Coenye, 2004)	DSMZ (11853)

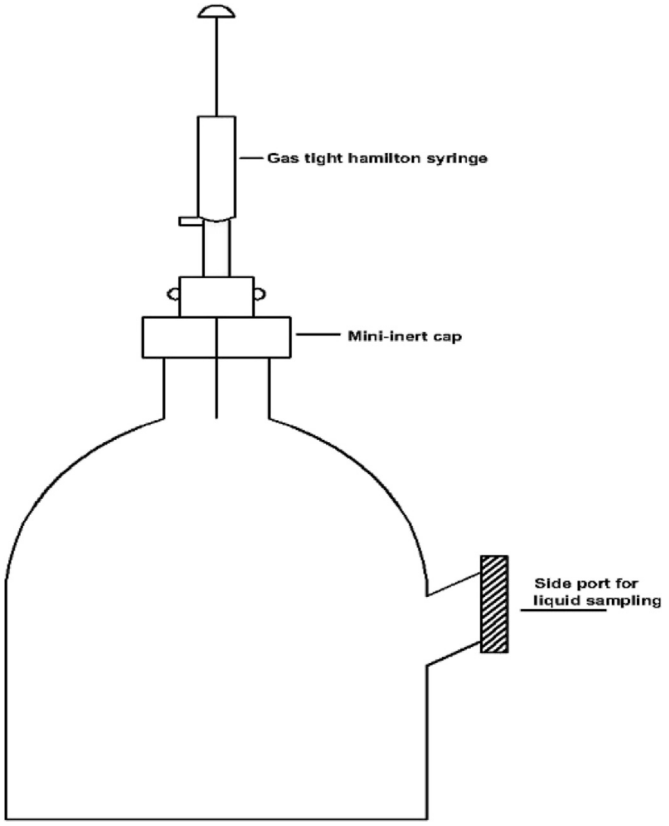

Fig. 1. Illustration of the glass bottle reactor used to conduct biodegradation studies of test chemicals. 200 mL of sterile mimimal medium was used for the experiment.

wrapped in aluminium foil before being placed on a shaker table for 2 min to homogenize the reactor contents. All biodegradation assays were run in triplicate. An abiotic control was prepared in a similar way but without the inoculum. Reactors were incubated at 22 °C and shaken at 155 rpm in an incubator (Multitron Pro, INFORS HT, UK). 1.5 mL of liquid sample was collected from the side port using a sterile syringe (Syringe Discardit II 2 mL; VWR, United Kingdom) and needle (VWR, United Kingdom). 1 mL samples (except for the sample from the reactor with toluene) were clarified by centrifugation (8000×*g*, 5 min) followed by filtration (0.2 µm PVDF syringe filter; VWR, United Kingdom). 0.5 mL of filtrate was mixed with 0.5 mL methanol (Sigma-Aldrich, United Kingdom) and analysed to quantify the chemical removal by HPLC (Section 2 **in SI;** Table S1) (Shimadzu, UK). Toluene was extracted in hexane (Sigma-Aldrich, United Kingdom) using a liquid-liquid extraction method and the hexane extract was analysed by GC-FID (Agilent Technologies, Palo Alto, USA) according to the method described in **SI (**Section 3**).** Furthermore, headspace toluene in the reactor was also quantified over the duration of the experiment by GC-FID (Section 3 **in SI).** The remaining cell solution was resuspended, 0.5 mL of which was analysed using a flow-cytometer (Becton Dickinson, California) to quantify cell growth in the reactors (Section 4 **in SI**). The headspace CO_2 development during the experiment was quantified by GC-MS (**Section 5 in SI**).

2.3. Carbon mass balance

A mass balance for carbon was carried out for each batch reactor of pure degrader culture; the initial and final total carbon content were determined. The total carbon in the reactor consisted of carbon present in the biomass, the headspace and the dissolved substrate, and headspace and dissolved CO_2 (including carbonate and bicarbonate). The carbon content of the biomass was estimated using the carbon content per bacterial cell (i.e. 9.4×10^{-14} g C/cell) mentioned elsewhere (Vrede et al., 2002) according to Equation (6.18) (**Section 6 in SI**). The total CO_2 carbon content in the batch was estimated using Equation (6.7) **(Section 6 in SI)** which is based on carbon dioxide and carbonic acid-base equilibria (Stumm and Morgan, 1970).

A theoretical carbon mass balance for each chemical mineralization was also performed. To achieve this, stoichiometric equations for microbial growth from chemical biodegradation were developed, with the assumption that NH_4^+ was the sole nitrogen source, using the method suggested by McCarty (2012). Briefly, bacterial growth involves two basic reactions: one for energy production and one for cellular synthesis. The electron donor provides electrons to the electron acceptor for energy production. A portion of its electrons (f_e) is transferred to the electron acceptor to provide energy for the conversion of another portion of electrons (f_s) into microbial cells. On a net yield basis, an assumption was made that 40% of the electron equivalent in electron donor substrate is used for synthesis (f_s=0.4) (McCarty, 2012), while 60% is used for energy (f_e=0.6). Then, the overall energy and synthesis reactions were developed using the half reaction approach as described in **Section 9 in SI**.

2.4. Modelling biodegradation and estimation of kinetic parameters for pure culture experiments

For simplicity, most QSRB models are based on the concept of first-order biodegradation kinetics which relate the substrate (or chemical) half-life, $t_{1/2}$ (h), or similar empirical observations, to a first-order biodegradation rate constant, k_{deg} (h^{-1}).

$$k_{deg} = \frac{\ln(2)}{t_{1/2}} \quad [1]$$

This approach ignores the dependency of the biodegradation process on the concentration of substrate utilizing biomass in the system. The change in the substrate concentration S (moles C substrate.m^{-3}) is simply described by

$$d/dt\, S = -k_{deg} \cdot S \quad [2]$$

However, as a minimum, a realistic model of microbial processes should relate changes in the active biomass B (mole C biomass.m^{-3}) in the system to the utilization of the primary substrate S that limits

the growth of the biomass. The relationship most frequently used is the model of Jacques Monod (Pitter and Chudoba, 1990; McCarty, 2012), which relates the specific growth rate of bacteria, μ (h^{-1}), and specific substrate utilization/removal rate, q (mole C substrate.mole C biomass^{-1}.h^{-1}), as follows:

$$\mu = \mu_{max} \cdot \frac{S}{K_s + S} \quad [3]$$

$$q = \frac{\mu_{max}}{Y} \cdot \frac{S}{K_s + S} = q_{max} \cdot \frac{S}{K_s + S} \quad [4]$$

$$q_{max} = \frac{\mu_{max}}{Y} \quad [5]$$

$$d/dt\, B = \mu \cdot B = \mu_{max} \cdot \frac{S}{K_s + S} \cdot B \quad [6]$$

$$d/dt\, S = -q \cdot B = -q_{max} \cdot \frac{S}{K_s + S} \cdot B \quad [7]$$

Where μ_{max} = maximum specific growth rate (h^{-1}), K_s = substrate saturation constant (moles C substrate m^{-3}) (i.e. substrate concentration at half μ_{max}), q_{max} = maximum specific substrate utilization or removal rate (mole C substrate.mole C biomass^{-1}.h^{-1}), Y = yield coefficient (mole C biomass.mole C substrate^{-1}).

In this study, the batch study data (i.e. substrate removal, biomass growth and headspace CO_2 development with time) was used to estimate kinetic growth parameters (μ_{max}, Y and K_s) for each chemical degraded. These parameters were estimated by fitting the experimental data with a carbon mass balance model simulating the pollutant biodegradation according to Monod kinetics (Simkins and Alexander, 1984). The model evaluates the agreement between predictions and data for a given Y (determined from the end-point biomass and CO_2 data) and a range of μ_{max} and K_s parameter value combinations, considering substrate, biomass and CO_2 data, and identifies as the best-fit model parameters those that give the minimum sum of squared residuals for the combined data. The model also includes a biodegradation lag phase, t_{lag} (h), as an option. The parameter q_{max} was calculated according from Equation (5). The model was implemented in Matlab©, and the underpinning equations and parameters are provided in the **SI (Section 6 and 7**).

2.5. Statistical analysis

All statistical analysis were performed using Excel. The coefficient of determination (R^2) and its significance was obtained from a simple linear regression model to determine the relationship between q_{max} and q_{max}/K_s and first-order biodegradation rate constants, k_{deg}, predicted by a previously proposed QSBR model (Acharya et al., 2019). A univariate regression analysis between q_{max} and molecular descriptors characterizing the chemicals in a previous QSBR study (Acharya et al., 2019) was also performed.

3. Results and discussion

3.1. Batch biodegradation of chemicals

The concentration profiles of biomass, chemicals and CO_2 during aerobic biodegradation of five different chemicals at a fixed initial concentration (i.e. 50 mg/L) are shown in Fig. 2 and **SI (Section 13;** Fig. S3**)**. Biodegradation of phenol, toluene and m-cresol proceeded without any lag phase, while lag phases were observed for 4-chlorophenol and 2,4-dichlorophenol biodegradation. The biodegradation of test chemicals predominantly occurred during the exponential phase of microbial growth, as is commonly observed (Reardon et al., 2000). This can be explained by the abundance of space, nutrients and desired conditions for growth (Pitter and Chudoba, 1990; Rolfe et al., 2012) immediately following the lag phase, allowing microorganisms to grow at their maximum rate. All chemicals were mineralised, as illustrated by a continual decrease in the chemical concentration and increase in the headspace CO_2 and biomass concentration. Furthermore, biomass growth in all the reactors was mirrored by chemical, i.e. carbon substrate, consumption (substrate utilization). The time required for complete biodegradation of each chemical differed **(**Fig. 2 **and Section 13 in SI;** Fig. S3**).** In all reactors, there was no significant difference between the initial and final pH values (two sample *t*-test, p-value > 0.05), since a pH buffer had been added to the growth medium. In the control experiments (results not shown) conducted in the absence of the inoculum, no change in the concentration of chemicals was observed, indicating abiotic removal processes were insignificant.

3.2. Monod kinetics parameters

The different components and parameters associated with test chemical biodegradation in the batch reactors are presented in Fig. S2 **in SI** along with the Monod kinetic model predictions, which show how the best fit kinetic parameters for each chemical degradation were estimated. All the components are expressed in moles carbon per m^3 to enable a carbon mass balance. Examination of Fig. S2 **(Section 10 in SI)** suggests that the Monod model provided a reasonable fit to the acquired experimental data, especially for substrate removal. However, the fit for CO_2 was not so good. This could be attributed to a CO_2 yield that varies as a function of time at different stages of the bacterial growth. The experimental data sometimes appears to show two phases of biodegradation, demonstrating more complexity in the biodegradation process than considered by Monod. This might be due the partial degradation of substrate initially to a metabolite, so there is a delay between the substrate degradation and the fully equivalent biomass and CO_2 production. On the other hand, the assumed partitioning equilibrium between CO_2 in headspace and aqueous medium may not always be instantaneous as assumed by the model.

To obtain a model fit, good knowledge of initial values for Monod parameters was required, as convergence can often not be readily achieved in the data fitting (Zhang and Hughes, 2004). It should be noted that the range of fitting parameters (i.e. μ_{max} and K_s) used in the model was based on the initial fitting parameters calculated with experimental biodegradation results using a method mentioned elsewhere (Simulator, 2018). However, when fitting the Monod model directly to experimental data, K_s values varied with μ_{max}, indicating optimization of the two parameters was not completely independent, rather they draw each other during the fitting (Kovárová-Kovar and Egli, 1998). In fact, a fixed ratio of μ_{max} to K_s tends to give very similar agreements between measured and modelled data.

Table 2 presents the various kinetic and biological parameters obtained from the modelling of the biodegradation of each of the test chemicals in closed reactors. The value of the maximum specific growth rate (i.e. μ_{max}), which describes the biomass growth under ideal conditions, was slightly higher for *Pseudomonas putida* F1 when toluene (0.888 h^{-1}) was used as a substrate followed by m-cresol (0.755 h^{-1}) and phenol (0.666 h^{-1}). Whereas, the μ_{max} value for *Cupriavidus baselensis* was higher when 4-chlorophenol (0.266 h^{-1}) was used as substrate compared to 2,4-dichlorophenol (0.036 h^{-1}). The two organisms used in the biodegradation assays are different and may have different

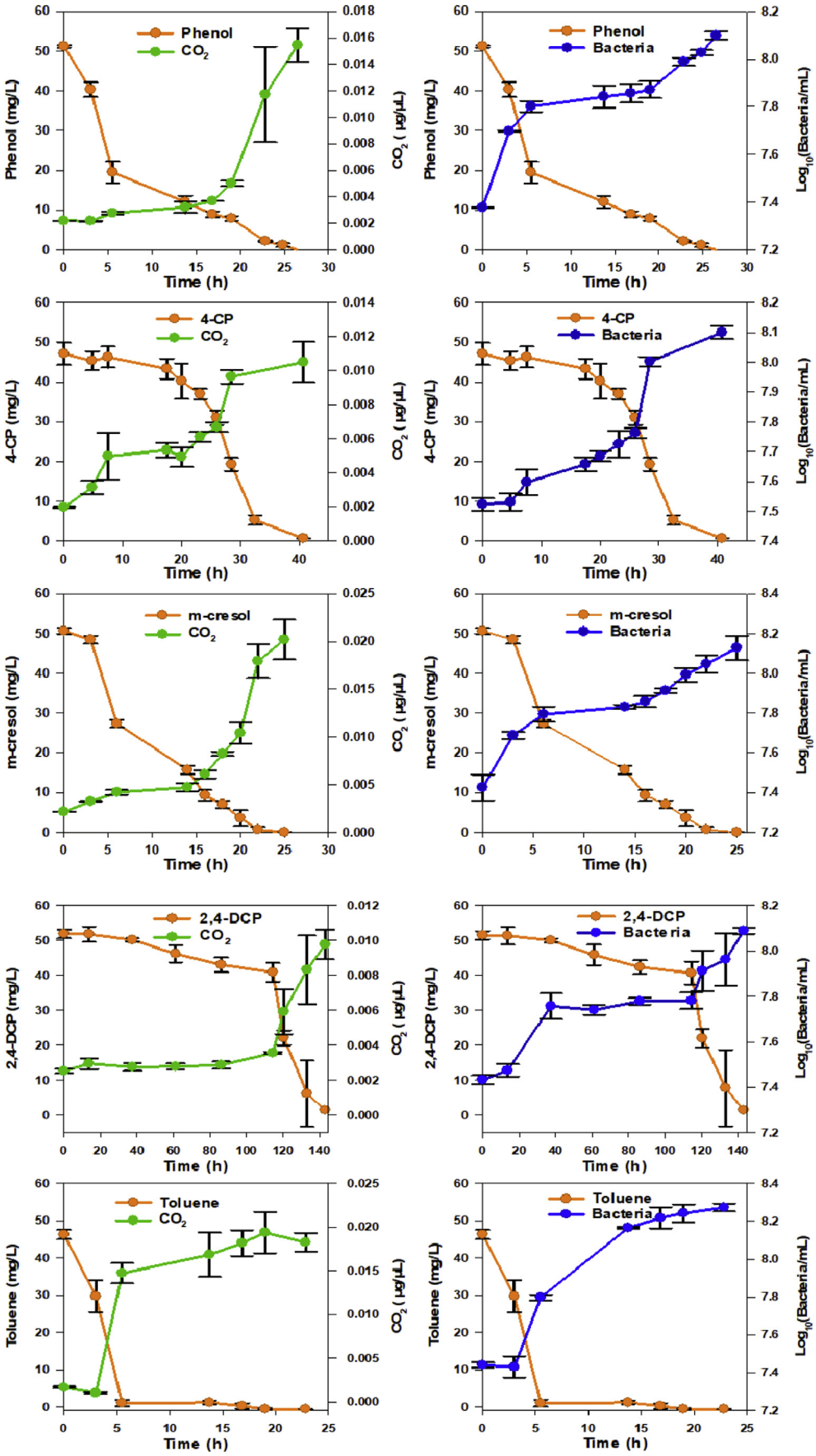

Fig. 2. Chemical concentrations, biomass growth and headspace CO_2 concentration profiles obtained during biodegradation experiments with known degraders at room temperature for phenol, 4-Chlorophenol, m-cresol, 2,4-DCP and toluene. Error bars represent the standard deviations for the triplicate experiments and might not be visible in some cases.

transport and metabolic efficiencies. However, studies have shown that, when glucose was used as a sole carbon source in mineral medium, *Pseudomonas putida* F1 and *Ralstonia eutropha* (95.7% homology (i.e. sequence similarity) with *Cupriavidus baseliensis* (formerly *Ralstonia* sp strain RK1) (Steinle et al., 1998; Vandamme and Coenye, 2004)) have maximum specific growth rates (i.e. μ_{max}) of 0.20 h^{-1} (Oliveira et al., 2009) and 0.23 h^{-1} (Marangoni et al., 2001), respectively, indicating a similar metabolic efficiency for at least glucose degradation.

The kinetics of microbial growth under limiting substrate conditions, the scenario commonly prevalent in the environment, is assessed by the ratio of μ_{max} and K_s (i.e. μ_{max}/K_s), also termed specific affinity (Kovárová-Kovar and Egli, 1998). This ratio reflects the competitiveness of a microbial population to grow on a low concentration of limiting resource: the higher the value of μ_{max}/K_s, the better the microbial population grows on a limiting resource resulting in rapid substrate depletion (Healey, 1980). The specific affinity, μ_{max}/K_s, obtained for test chemicals (Table 2) showed that at low substrate concentration, microbial growth on toluene was the fastest, followed by phenol, m-cresol and 4-chlorophenol and finally 2,4-dichlorophenol. For toluene, it was noted that the fitted value of μ_{max}/K_s, was strongly dependant on the measurements at time 5.5 h, as elimination of this single time point from the set altered the fitted K_s, and μ_{max}/K_s, by a factor of 2 (Table 2). The measured concentrations at time 5.5 h look somewhat suspicious, as there appeared to be no further reduction in the substrate concentration until time 13.75 h but a small amount of CO_2 production and significant biomass growth during that same time interval (Fig. 2). Hence, while the toluene μ_{max} parameter value appears to be robust, there is considerable uncertainty about the correct toluene parameter values for K_s, and μ_{max}/K_s, with implications for the toluene substrate utilization rate at low substrate concentration, as will be discussed further below.

The yield coefficient for the biodegradation of each chemical was measured using the experimental results, which is the mass of biomass formed per unit mass of chemical consumed at the end of the experiment (Table 2**)**. Yield coefficient data revealed that *Pseudomonas putida* F1 can produce higher amounts of biomass carbon per unit mass of toluene carbon than per unit masses of phenol and m-cresol carbon. This is in agreement with the pattern observed in the case of aerobic biodegradation of toluene and phenol by *Pseudomonas putida* F1, where the higher yield coefficient was achieved for toluene at 30 °C (Reardon et al., 2000). 4-chlorophenol and 2,4-DCP biodegradation data revealed that, for *Cupriavidus baseliensis,* the efficiency was better for 2,4-dichlorophenol than for 4-chlorophenol.

The kinetics of substrate utilization under ideal conditions are described by q_{max}. The pattern of q_{max} for the studied chemicals was m-cresol > phenol > toluene> 4-chlorophenol > 2,4-dichlorophenol. However, q_{max} for toluene, m-cresol and phenol were similar to each other, and higher than for 4-chlorophenol and 2,4-dichlorophenol. The value of q_{max}/K_s relates to the substrate utilization at low substrate concentration. The pattern of q_{max}/K_s for the studied chemicals was toluene > phenol > m-cresol > 4-chlorophenol > 2,4-dichlorophenol. However, there is uncertainty around the parameter value for the ratio q_{max}/K_s of toluene, due to the same issues as were discussed above for the ratio μ_{max}/K_s.

3.3. Carbon mass balance and stoichiometry to assess compatibility of data sets in the biodegradation experiments

Complete degradation of test chemicals was observed, although the time required for complete degradation differed for each test chemical. Fig. 3 presents the carbon mass balance of the test chemical biodegradation in the reactors. The mass balance showed an average recovery of 80.7% of the initially applied carbon, ranging from 77.9% to 81.5% recovery, depending on the test chemical. Variation between replicates for carbon mass balance recovery was typically low. The ability to account for 80% of the introduced carbon in reactors provided an opportunity to determine the fate and partitioning of the test chemicals during the biodegradation process, either to inorganic carbon (CO_2) or its incorporation within the biomass. The production of metabolities is a significant phenomenon in the biodegradation of chemicals (Martin et al., 2017b) and can potentially account for the remaining fraction of missing carbon in the mass balance. Despite this, the phenomenon is probably insignificant in this study. Oxygen availability was not limiting so that complete mineralization of each chemical in the biodegradation tests was possible (**Section 12 in SI;** Table S5). In addition, thermodynamic analysis suggested that the complete biodegradation of each chemical was feasible (**Section 9 in SI**). Furthermore, the absence of leaks from the reactor glass bottle **(Section 8 in SI;** Fig. S1), ensured that observations were not confounded by potential losses through volatility or leakage. However, uncertainty in the Henry constant and the cell carbon content value used in the model could have a significant effect on the calculation of the final carbon content of the reactor. In our study, a cell carbon content value of 9.4×10^{-14} g C/cell (Vrede et al., 2002) was used, and biomass carbon on average accounted for 43% of the carbon in the final amount of total carbon. In general, the elemental composition and cell volume of bacteria varies among species and is also influenced by environmental conditions (Trousselier et al., 1997; Vrede et al., 2002; Elazhari-Ali et al., 2013). Previous studies have shown that the elemental content of bacterial cells varies across the different phases of growth: the highest carbon content was observed in exponentially growing cells, whereas the lowest was observed when the substrate starts to get limited (Vrede et al., 2002). Carbon content per cell was therefore also estimated in

Table 2
Estimated Monod model parameter values for aerobic biodegradation of the test chemicals by known degraders. The numbers in the parenthesis represent the kinetic parameters for toluene, when biodegradation data (i.e. Substrate, CO_2 and biomass concentration) from the third time point (i.e. t = 5.5 h) were excluded during the model fitting.

Chemical	[a]Yield Coefficient [Y]	[b]K_s	Maximum specific growth rate[c] [μ_{max}]	μ_{max}/K_s	q_{max} [d]	$\mathbf{q}_{max}/K_s$
Phenol	0.244	10	0.666	0.066	2.730	0.273
m-cresol	0.275	13.79	0.755	0.054	2.743	0.198
toluene	0.379	3.33 (6.71)	0.888 (0.844)	0.266 (0.125)	2.344 (2.228)	0.703 (0.349)
4-CP	0.352	7.5	0.266	0.035	0.758	0.101
2,4-DCP	0.397	2.14	0.036	0.016	0.090	0.042

The unit of K_s,Y, q_{max}, μ_{max} and q_{max}/K_s are (mole C substrate.m^{-3}), (mole C biomass. mole C substrate^{-1}), (mole C substrate. mole C biomass^{-1}.h^{-1}), (h^{-1}) and (m^3.mole C biomass^{-1}.h^{-}1), respectively.

[a] Yield coefficient: mass of biomass C produced per unit mass of substrate C consumed during each chemical biodegradation.

[b] K_s and.

[c] μ_{max}: best fit parameters obtained by fitting the biodegradation data with the Monod model q_{max}.

[d] Calculated with Equation (4).

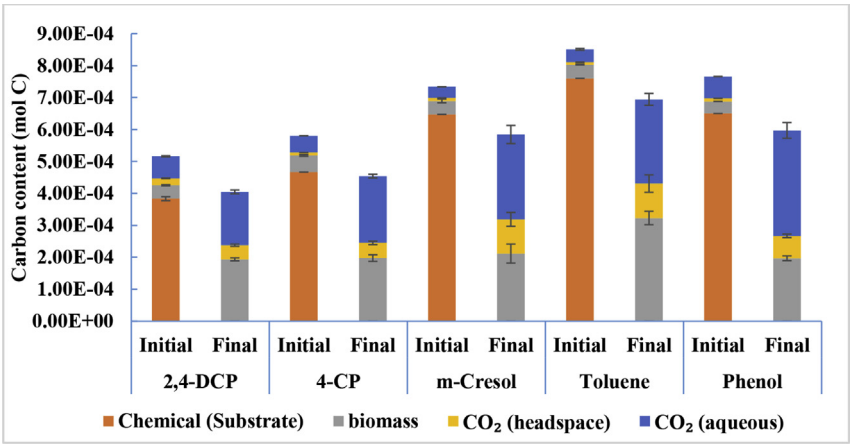

Fig. 3. Clustered stacked column chart summarizing the mass balance for each test chemical biodegradation. Error bars represent the standard deviations of carbon content for each component mentioned in the figure legend. Experiments were run in triplicate.

Table 3
Comparison of theoretically estimated biomass and CO_2 amount for each test chemical biodegradation with the experimentally observed amounts. A theoretical carbon mass balance for each chemical mineralization was also performed with stoichiometric equations for microbial growth using the method suggested by McCarty (2012) [Section 9 in SI].

	Theoretical Yield		Experimental Yield	
	[a]CO_2	[b]Biomass	[a]CO_2	[b]Biomass
Phenol	0.533	0.467	0.6 ± 0.055	0.251 ± 0.012
m-Cresol	0.514	0.486	0.649 ± 0.075	0.218 ± 0.013
Toluene	0.486	0.514	0.539 ± 0.037	0.326 ± 0.019
4-Chlorophenol	0.567	0.434	0.519 ± 0.039	0.302 ± 0.021
2,4-Dichlorophenol	0.600	0.400	0.481 ± 0.043	0.411 ± 0.017

[a] moles of CO_2 carbon produced per mole of substrate carbon.
[b] moles of biomass carbon produced per mole of substrate carbon.

this study after complete degradation of the chemicals by assuming that all the substrate was converted into biomass and CO_2, that the phase partitioning and CO_2 speciation in the aqueous medium was accurately described, and that CO_2 was measured accurately (Refer **Section 14 in SI** for calculation). We observed different carbon contents per cell in *Pseudomonas putida* F1 for the degradation of different test chemicals: the highest values were observed during m-cresol (1.84×10^{-13} g C/cell) degradation compared to phenol (1.75×10^{-13} g C/cell) and toluene (1.46×10^{-13} g C/cell) degradation. Similarly, the carbon content per cell for *Cupriavidus baselensis* during 2,4-dichlorophenol and 4-chlorophenol degradation were 9.11×10^{-14} and 1.31×10^{-13} g C/cell, respectively. Therefore, mass balance data would potentially be more rigorous if carbon content of different strains can be determined experimentally for each chemical biodegradation test. However, quantifying the cell carbon content of individual bactaria was outside the scope of this study. On average, 65% of test chemical was mineralised to CO_2, ranging from 53.9% to 74.8%, depending on the test chemical (Table 3). Whereas, 35% of test chemical carbon was incorporated into biomass (ranging 25.1%–46%) (Table 3). The amount of substrate carbon going to biomass reported in the literature varies considerably, ranging from 25% to 45% (Zhang and Hughes, 2004; Elazhari-Ali et al., 2013). It should be noted that a major proportion of nutrients taken up by bacteria are normally used for energy generation, simply to maintain cellular function (e.g., maintain membrane potential, maintain an osmotic pressure across bacterial membrane, synthesizing building blocks, take up or excrete some compounds against the concentation gradients) (Hu, 2017).

In this study, a stoichiometric equation was developed (**Section 9 in SI**) for the biodegradation of a series of test chemicals and the theoretical yield (biomass and CO_2) was compared with experimentally observed results. Table 3 shows the theoretically estimated and experimentally observed CO_2 and biomass amount for each test chemical. The average theoretical amount of biomass formation and CO_2 production were 0.46 mol of biomass C/mole of substrate carbon and 0.54 mol of CO_2 C/mole of substrate carbon respectively, ranging from 0.4 to 0.514 mol of biomass C/mole of substrate carbon and 0.486–0.6 mol of CO_2 C/mole of substrate carbon, depending on the test chemical. The average experimental amount for biomass and CO_2 formation were 0.3015 mol biomass C/mole of substrate carbon (range: 0.218–0.411 mol of biomass C/mole of substrate carbon) and 0.557 mol of CO_2 C/mole of substrate carbon (0.481–0.649 mol of CO_2 C/mole of substrate carbon) respectively, depending on the test chemical. Thus, the experimentally observed biomass formation was typically lower than the theoretically estimated biomass formation except for 2,4-DCP biodegradation, although the range of values measured encompasses the range of theoretical predictions. It has to be noted that the theoretically estimated yield (i.e. true yield) is based on the material balance equation between cells, substrate and the products and excludes biomass loss due to cell decay or endogenous respiration, and also the energy consumed by cells for the maintenance, and is therefore always higher than net yield (i.e. experimentally observed yield).

3.4. Verification and calibration of a QSBR model

In the environment, the rate of chemical biodegradation is uncertain and difficult to reproduce as it is influenced by spatial and temporal variability of a combination of different factors (i.e. abundance and activity of degrading microorganism, environmental conditions, structure and concentration of chemicals) (Pavan and Worth, 2008). In contrast, the different parameters influencing biodegradation can be more controlled in laboratory biodegradation tests. Particularly, the biodegradation rates obtained from simplified biodegradation system (i.e. batch biodegradation assays, where the chemical acts as the only source of carbon and energy) are easily interpretable and comparatively reproducible as compared to biodegradation rates obtained from mixed culture assays. Batch biodegradation studies might therefore be suitable towards calibrating theoretical QSBR models. However, it must be noted that several factors can effect degradation of

chemicals in pure culture studies, which could ultimately have influence on the estimated biodegradation rate and thus on calibration of QSBR models. For example, culturing degraders in nutrient broth media prior to inoculating them into the mineral medium with a specific chemical might contribute in the possible momentary loss in the biodegradation capacity of the degrader. Therefore, a lag phase can be observed during the biodegradation assays, whereby bacteria take time to adapt to the new environment, which can include the induction of enzymes needed for catalyzing degradation reactions.

Direct comparison of the first order rate constants (i.e. k_{deg}) used in the previous QSBR study (Acharya et al., 2019) and experimentally determined substrate utilization rates (i.e. q) from this study for selected test chemicals may be misleading, as there is no direct equivalence between the Monod kinetic model parameters, which link the substrate utilization to the growth of biomass, and first-order biodegradation rate constants, which ignore the dependency of the rate on the growth of biomass during the biodegradation assay. The previous rate constants were derived from the BIOWIN3 model (pass/fail model for biodegradibility), and transformed into rate constants that do not reflect those measured experimentally in this study. On the other hand, the majority of existing QSBRs, including that in our previous study, are developed with rate constants from different biodegradation databases, which are often based on another form of QSBR. The chemical biodegradation rate constants used in the development of such databases are derived from first tier biodegradation screening tests (typically OECD 301 or 302 tests) and rarely represent the true rates. The reliability of the predicted chemical degradation rate constant (in different environmental compartments) with existing QSBR models is a major concern (Peijnenburg and Damborský, 2012). This demands a method/approach that can be used to validate and/or calibrate the existing models, so that it is possible to apply the existing QSBR models to predict the chemical degradation rate constant in any environmental compartment.

Biomass growth is fueled by substrate utilization and therefore the rate of substrate utilization, or removal (i.e. q in Eq (4)) is regarded as the basic rate in the biodegradation process (McCarty, 2012). Hence, correlation analysis between the first-order biodegradation rate constant of the QSBR models and the experimentally determined substrate utilization rates in this study are of interest. From Eq (4) it can be seen that, at high substrate concentration, the utilization of different substrates can be compared based on the maximum substrate utilization rates, q_{max}. At low substrate concentration, the utilization of different substrates can be compared based on the ratios q_{max}/K_s.

A strong and significant correlation ($p < 0.05$, $r^2 = 0.85$), along with similar patterns, were observed between the q_{max} and those first order biodegradation rate constants, k_{deg}, from previously developed QSBR models for five of the test chemicals (Fig. 4A). Similarly, the correlation between q_{max}/K_s and the first order biodegradation rate constants, k_{deg}, for 4 chemicals were also significant (p-value <0.05, $r^2 = 0.97$) (**Fig. 4B**). Due to the uncertainty in the q_{max}/K_s parameter value of toluene, which were discussed above, toluene was not included in this regression analysis. If toluene is included with a q_{max}/K_s ratio of 0.703 (Table 2), the correlation coefficient r^2 would be 0.245 (p-value > 0.05), and the toluene data point would be a clear outlier in the data set (see Fig. S6). If toluene is included with a q_{max}/K_s ratio of 0.349 (Table 2), the r^2 would be 0.71 (p-value < 0.05).

Even though the biodegradation modelling concepts differ significantly between first-order rate kinetics and Monod kinetics, any of the rates determining parameters compared in Fig. 4 reflects the relative biodegradability of the different compounds, and this can explain the observed correlations. However, further validation and calibration of the principles behind *in silico* QSBRs with biodegradation rates obtained from a mixed microbial community, for example activated sludge (a similar type of inoculum as used in RBTs) are needed. In addition, it would be useful to extend such validations for biodegradation rates from a broad range of aromatic chemicals with different types of substituent groups, including non-halogenated moities.

Another fundamental way in which the principles of previously derived QSBR models (Acharya et al., 2019) could be verified is through correlation analysis between the molecular descriptors from those QSBR models and the experimentally determined parameters in this study (Table 4). We therefore also tested the statistical association between q_{max} of the five chemicals obtained from pure culture degrader experiments and each of the molecular descriptors by conducting univariate regression analysis (Table 4 and Table S4 in SI for details). In short, reported correlation coefficients revealed a significant correlation between q_{max} and most of the descriptors listed in the previous study for monoaromatic chemicals, some at p-value < 0.05 and most at p-value < 0.1. Because of the small number of samples, it was not feasible to perform multivariate regression using combinations of two or three descriptors. It is interesting to note that molecular descriptors given in Table 4 represent a variety of physicochemical, structural and quantum mechanical properties of chemicals that have previously shown an association with biodegradation rates (Okey and Stensel, 1993, 1996; Yang et al., 2006). More specifically, enzyme binding, chemical transformation, kinetics and thermodynamic factors are pivotal during the biodegradation of chemicals, as discussed elsewhere (Parsons and Govers, 1990; Pitter and Chudoba, 1990;

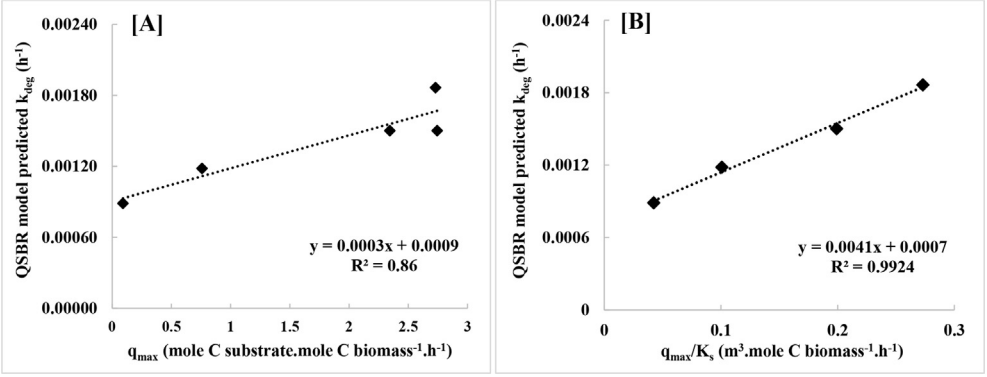

Fig. 4. Relationship between the 1st order biodegradation rates used in previously developed QSBR models and [A] maximum specific substrate utilization rates (q_{max}), or [B] q_{max}/K_s. Toluene was not included in the regression analysis between 1st order biodegradation rates and q_{max}/K_s due to the uncertainty in the parameter value.

Table 4
Summary table for univariate regression analysis values. The reported values are correlations between the observed maximum specific substrate utilization rate of test chemicals and the individual molecular descriptors from QSBR models developed previously (Acharya et al., 2019).

	[a]Mor08u	[b]nArX	[c]tot.energy	[d]E $_{homo}$	[e]E $_{lumo}$	[f]Elec. energy	[g]MW	[h]vdw V	[i]MR	[j]Log P	[k]σ	[l]$_vX^1$	[m]$_vX^2$
R^2	−0.272	0.920	0.589	0.775	0.835	0.618	0.806	0.546	0.540	0.416	0.689	0.623	0.613
Std. Error	1.385	0.348	0.788	0.582	0.500	0.759	0.541	0.828	0.833	0.939	0.685	0.755	0.764
p-value	0.730	0.006	0.081	0.031	0.019	0.072	0.025	0.095	0.097	0.145	0.052	0.070	0.073

[a] Un-weighted descriptor with scattering parameter (s) = 7 $Å^{-1}$ (3D Molecular Representations of Structure based on Electron diffraction).
[b] Number of halogen on aromatic rings (Functional group counts).
[c] Total energy (ToE) of molecule.
[d] Energy of highest occupied molecular orbital.
[e] Energy of lowest occupied molecular orbital.
[f] Electrical energy of molecule.
[g] Molecular weight.
[h] van der Waal Volume of molecule.
[i] Molar Refractivity.
[j] n-octanol and water partition coefficient.
[k] Substituent (hammett) constant.
[l] 1st order valence connectivity index.
[m] 2nd order valence connectivity index.

Wammer and Peters, 2005; Arnosti, 2011; Acharya et al., 2019). Briefly, the observed statistically significant correlation (p-value <0.1) between the q_{max} and hammett (substituent) constant suggests that the rate limiting steps in the chemical transformation reaction of an aromatic chemical is an electrophilic substitution. In such cases, the chemical reaction is slowed down when the electron density of the reaction centre is reduced by the substituents possessing positive values (e.g., halogen and nitro substituents) on the substituent constant (Pitter and Chudoba, 1990). Conversely, the reaction rate is accelerated by electron-donating or activating groups, such as hydroxyl or methyl groups (Pitter and Chudoba, 1990). The results in this study are in agreement with the results demonstrated by Alexander and Lustigman (1966), Martin et al (2017a,b) and Pitter and Chudoba (1990). Alexander and Lustigman (1966) showed slower rates of biodegradation of mono- and di-substituted benzene by soil microorganisms with chloro-, nitro- and sulfonate-substituents, whereas an increased rate was recorded in the presence of hydroxyl and carboxyl groups. Similarly, Martin et al. (2017a) showed that, phenol substituent groups at position 3 (meta-position) have significant effects on the biodegradation potential of chemicals, where the sequential order of biodegradation by substituent groups was carboxylic acid ($COOH$) > hydroxyl (OH) > methyl (CH_3) > methoxy (CH_3O) > chloro (Cl) > nitro (NO_2) > bromo (Br) > fluoro (F). Likewise, a similar trend was observed in a study performed by Pitter and Chaudoba (Pitter and Chudoba, 1990). Furthermore, the statistically significant (p-value < 0.1) correlation between q_{max} with steric (vdw V, MR and MW) and topological ($_vX^1$ and $_vX^2$) descriptors, indicates the importance of chemical fit in to the enzyme active site during biotransformation of chemicals. In most chemical reactions, an energy barrier exists that must be surmounted for the reaction to occur (Wammer and Peters, 2005). Descriptors like total energy, electrical energy and E_{homo} are quantum chemical descriptors that generally provide information on energy associated with a chemical entity (Parsons and Govers, 1990). The significant correlation (p-value <0.1) between q_{max} with quantum chemical descriptors suggests that the kinetics of biotransformation of aromatic chemicals is slower when the value of these quantum chemical descriptors is higher. For example, the higher the E_{homo} (Energy of highest occupied molecular orbital) for a particular chemical, the higher the amount of energy needed during the chemical reaction to remove an electron from the molecule, which ultimately slows down the chemical reaction. Similarly, if the electrical energy and total energy of the molecule is high, there is strong attraction between electrons and the atomic nuclei. This implies that, high energy is required to degrade such a molecule, which as a consequence is more resistant to degradation than a molecule with lower electrical and total energy. Furthermore, the q_{max} values were not correlated with the lipophilic parameter (i.e. LogP of the test chemicals), suggesting that at high substrate concentration, lipophilicity might not be the rate limiting factor. Thus, all together these results suggest that the rate of biodegradation of mono-aromatic chemicals seems to be governed by the electronic effects of substituents, kinetic and thermodynamic factors associated with chemical biotransformation, and the shape and size of the chemicals.

4. Conclusions

- The biodegradation experiments including a rigorous carbon mass balance and theoretical yield predictions enabled the generation of well substantiated substrate utilization rates and related parameters q_{max} and q_{max}/K_s.
- Experimentally determined q_{max} and q_{max}/K_s can be used to validate and calibrate the principles behind *in silico* QSBRs.
- The substrate utilization rates derived from pure culture experiments demonstrate that the same rank prioritisation existed with biodegradation rates derived from ready biodegradability tests, even if the rate metrics and values were different.
- The simple but rigorous experimental and theoretical approach used in this study could form the basis towards calibrating the QSBR models with real biological data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.05.075.

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